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Cloning of *Bacillus licheniformis* Xylanase Gene and Characterization of Recombinant Enzyme

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Abstract Hemicellulose is a major component of lignocellulose biomass. Complete degradation of this substrate requires several different enzymatic activities, including xylanase. We isolated a strain of *Bacillus licheniformis* from a hot springs environment that exhibited xylanase activity. A gene encoding a 23-kDa xylanase enzyme, Xyn11, was cloned, and the recombinant protein was expressed in an *Escherichia coli* host and biochemically characterized. The optimum activity of the enzyme was at pH 5–7 and 40–50°C. The enzyme was stable at temperatures up to 50°C. Against birchwood xylan, the enzyme had an apparent K_m of 6.7 mg/mL and V_{max} of 379 μmol/min/mg.

Introduction

Lignocellulosic biomass is considered a prime alternative to fossil fuels as a source for many of our fuel and chemical feedstock needs. After cellulose, hemicellulose comprises the largest fraction of biomass [15]. Hemicellulose plays a role in crosslinking the other components of biomass via hydrogen and covalent bonds [5, 8, 18]. Effective hydrolysis of hemicellulose would both release this carbohydrate and increase access to the other components of lignocellulose. Thus, improved breakdown of hemicellulose is a key factor in maximizing biomass utilization.

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Hemicellulose is comprised primarily of xylan, a polymer of β-1,4-linked xylose residues [17]. The xylan provides attachment points for different chemical moieties, such as arabinofuranosyl and glucuronyl groups, that mediate linkages with the other components of lignocellulose [2, 3]. Xylanases are a class of enzyme that hydrolyzes the β-1,4 internal bonds of the xylan backbone polymer [13]. Based on the predicted three-dimensional structure of the catalytic residues, the xylanases can be categorized into glycosyl hydrolase (GH) families [6]. The majority of xylanases belong to one of two GH families: GH10 and GH11. GH10 xylanases are generally larger than those of GH11 and are better at hydrolyzing substituted xylan [4, 12]. However, GH11 xylanases usually have higher specific activity.

We have isolated a strain of xylanase-producing *Bacillus licheniformis* that was collected near a hot spring. We cloned a gene that was predicted to encode a family GH11 xylanase. Recombinant enzyme was produced and characterized in a bacterial host. The Xyn11 enzyme had an optimal activity at pH 5–7 and 40–50°C. When tested against a variety of substrates, the Xyn11 enzyme only had detectable activity against xylan substrates.

Materials and Methods

Cultures and Reagents

Samples of sediment containing mixed populations of microorganisms were collected from Borax Lake, Oregon hot springs (GPS coordinates N 42°20.129′ W 118°36.161′). *Escherichia coli* strain BL21(DE3)pLysE was obtained from Novagen (Madison, WI, USA). All DNA restriction endonucleases were obtained from New

England Biolabs (Ipswich, MA, USA). All chemicals were obtained from Sigma (St. Louis, MO, USA) unless otherwise specified.

Identification of *B. licheniformis* MS5-14

A pure culture of strain MS5-14 was isolated from the hot spring sample. Genomic DNA was prepared from MS5-14 using the FastDNA kit (Qbiogene, Irvine, CA, USA). The 16S rRNA gene was amplified from the genomic DNA by polymerase chain reaction (PCR) using the primers 27f and 1525r [9]. The 16S rRNA gene was cloned, and the sequence was compared to other genes in the GenBank database by BLAST (basic local alignment search tool) [1].

Cloning

Primers (bacX-5 and bacX-3) containing degenerate nucleotides were designed based on the conserved 5′ and 3′ ends of several *Bacillus* sp. xylanase genes. These primers were designed to incorporate the restriction enzyme recognition sites for *Nde*I and *Xho*I in the linker regions of the 5′ and 3′ oligonucleotides, respectively.

bacX-5: GCACATATGTTTAAGTTTAAAAAGAATT TYTTAGTTGGATTA
bacX-3: GCACGCTCGAGCCACACTGTTACGTTAG AACTTCCACTAC

The primers were used in PCR reactions to amplify the *xyn11* gene from *B. licheniformis* MS5-14 genomic DNA. PCR reactions were conducted in 50 μL [containing 100 ng genomic DNA template, 2.5 μM of each primer, 10 mM dNTP, and 1.25 U of PfuUltra Hotstart enzyme (Stratagene, La Jolla, CA)] using an initial denaturation step at 95°C for 2 min; 30 amplification cycles of 30 s at 95°C, 30 s at 53°C, 90 s at 72°C; and a final 10-mine extension at 72°C. The resulting PCR product and pET29b+ plasmid (Novagen) were digested with restriction enzymes *Nde*I and *Xho*I and ligated to produce pET29-MS14-X11 expression vector.

MS514-X11 Expression and Purification

The pET29-MS514-X11 plasmid was transformed into BL21(DE3)pLysE host cells. A liquid culture of the transformed colony was grown in Luria-Bertani (LB) broth by shaking at 37°C. Recombinant protein expression was induced by addition of isopropyl-*β*-D-thiogalactopyranoside to a final concentration of 1 mM when the optical cell density at 600 nm reached 1.5. After 3 h of additional growth, the bacteria were harvested by centrifugation and lysed with BugBuster reagent (Novagen) according to the manufacturer's protocol. The recombinant Xyn11 enzyme was bound to a HiTrap SP column (GE Healthcare, Piscataway, NJ, USA) in 50 mM sodium phosphate (pH 7.2) and eluted by sodium chloride gradient (20 mM to 2 M). Fractions containing the Xyn11 enzyme were pooled and applied to a HisTrap HP column (GE Healthcare) in 50 mM sodium phosphate (pH 7.2) and eluted by imidazole gradient (50–500 mM). Fractions containing purified enzyme were pooled and buffer-exchanged into 50 mM sodium phosphate (pH 7), 50 mM sodium chloride, and 10% glycerol using EconoPac 10DG desalting column (Bio-Rad, Hercules, CA, USA).

Enzyme Activity Assays

Xylanase activity on solid media was detected by visible clearings around microorganisms growing on 0.1% RBB-xylan (4-*O*-D-glucurono-D-xylan-remazol brilliant blue R), a dye-labeled substrate (Megazyme, Bray, Ireland). For hot spring environmental samples, microorganisms were spread on modified defined media agar plates (EZ- rich defined medium, Teknova, Hollister, CA, USA) containing 0.2% native xylan substrate (beechwood xylan, birchwood xylan, and oat spelt xylan at equal ratios) and 0.1% RBB-xylan. For BL21(DE3)pLysE transformants, the cells were spotted on LB agar plates containing 0.1% RBB-xylan.

Enzymatic activity against different substrates at various conditions was monitoring by measuring release of reducing sugar. In brief, 50-μL aliquots of the reaction were removed at various time points and added to 75 μL DNSA reagent (1% dinitrosalicyclic acid and 30% potasium sodium tartrate in 0.5 M sodium hydroxide). The samples were then heated at 100°C for 5 min and cooled, and the optical density was measured at 540 nm. Xylose solutions of known concentrations were processed in parallel as standards. Kinetic parameters were determined using the birchwood xylan substrate in concentrations ranging from 0.5 to 30 mg/mL. All liquid activity assay data are the results of three replicates.

Analysis of Xylanase Hydrolysis Products

Recombinant xylanase (92 nM) was incubated with 1% birchwood xylan in a 25-μL volume at 40°C for 18 h. Undigested xylan and enzyme were precipitated from the reaction by adding 75 μL ethanol, incubating on ice for 10 min, and collecting the supernatant. The liquid was then evaporated, and the reducing sugar ends were labeled with a fluorophore using the Carbohydrate Labeling and Analysis Kit (Beckman Coulter, Fullerton, CA, USA). The reaction products were then diluted 250-fold and analyzed on a P/ACE MDQ capillary electrophoresis system (Beckman Coulter). Separations were performed using a 40-cm uncoated fused-silica capillary column of 50 mm internal diameter (MicroSolv Technology Corp., Long

Branch, NJ, USA). Analyses were carried out at 25°C with an applied voltage of 15 kV using 100 mM sodium borate, pH 10.2, as the running electrolyte. A typical run schedule was 0.5 min at 50 psi in 0.1 N sodium hydroxide, 0.5 min at 50 psi in H_2O, 0.5 min at 50 psi in 0.1 N hydrochloric acid, 0.5 min at 50 psi in H_2O, 3.5 min at 50 psi in 100 mM sodium borate (pH 10.2), conditioning at 30 kV for 5 min, injection for 2 s at 0.2 psi, and separation at 15 kV. The detection system was a Beckman laser-induced fluorescence detector using an excitation wavelength of 488 nm and detection at 520 nm.

Results and Discussion

Identification of *B. licheniformis* Strain MS5-14

Sediment from an environmental sample collected from a hot spring (57°C) was used to inoculate a liquid defined media culture (Teknova) containing xylan as the carbon source and grown at 50°C. Samples from this culture were streaked onto minimal media agar plates containing RBB-xylan and incubated at 50°C. Cultures that produced clearings in the agar plate indicated xylanase activity and were isolated. One of those cultures was designated MS5-14. This strain formed off-white, smooth colonies with filamentous edges. The bacteria were short, slender motile rods that stained Gram positive.

To identify the organism, we cloned the 16S rRNA gene and compared the sequence to others in the database. The MS5-14 16S rRNA gene had very high identities (>99%) to the 16S rRNA from *B. licheniformis* ATCC 14580 type strain [14]. As such, we named the newly isolated culture *B. licheniformis* strain MS5-14.

Cloning of *MS514-xyn11* Gene

We aligned the nucleotide sequences of three *Bacillus* sp. xylanase genes that were in the GenBank database (accession Nos. DQ520129, M36648, and Z34519). We designed oligonucleotides against the conserved 5′ and 3′ ends of the genes. Although the sequence of the 3′ ends of the genes were identical, the 5′ ends of the genes had differences. Therefore, a degenerate oligonucleotide was designed against the 5′ end of the gene. Using the *B. licheniformis* MS5-14 genomic DNA as the template and the two primers, three independent PCR reactions were conducted to amplify the *MS514-xyn11* gene. The genes from each of the three PCR reactions were cloned separately and sequenced. The sequences of the genes from each of the independent PCR reactions were found to be identical including the region encompassed by the degenerate oligonucleotide.

The sequence of the *MS514-xyn11* gene (GenBank EU591524) was compared to those in the GenBank database. The *MS514-xyn11* gene was highly similar to that of *B. licheniformis* I5 (GenBank DQ520129), which was also isolated from a hot spring environment. At the amino acid level, the enzyme differed by only one residue with a Gln instead of a Pro at position 35. Based on the amino acid sequence, the enzyme was predicted to have a molecular weight of 23.4 kDa. The enzyme had a glycosyl hydrolase family 11 catalytic domain in region 32–212, and the highly conserved residues implicated to be critical for catalytic activity were present (Fig. 1) [7, 10, 16]. There was a potential signal peptide at the N-terminus. Two signal sequence analysis tools, SignalP-NN (neural network) and SignalP-HMM (hidden Markov model), predicted the cleavage site of the signal peptide to be either between residues 28 and 29 or between residues 23 and 24, respectively [11].

Biochemical Characterization of MS514-Xyn11

The *MS14-xyn11* gene was cloned into an expression vector, and the recombinant MS514-Xyn11 enzyme was produced and purified to apparent homogeneity from a bacterial host (Fig. 2a). The substrate specificity of the enzyme was tested against a variety of targets (Fig. 2b). MS514-Xyn11 had the highest activity against birchwood xylan. The enzyme had less (44%) activity against oat spelt xylan. MS514-Xyn11 had negligible activity against insoluble and soluble cellulose substrates (Avicel and carboxymethyl cellulose, respectively) and other glucose-based substrates (laminarin and lichenan).

The MS514-Xyn11 enzyme functioned between pH 5 and 7 with an optimum at pH 6 (Fig. 3a). The enzyme was most active between 40°C and 50°C (Fig. 3b). The thermostability of the enzyme was tested by preincubating the enzyme for 30 mins at various temperatures and then assaying for the remaining activity at 50°C (Fig. 3c). MS514-Xyn11 was stable at temperatures up to 50°C. However, at 55°C, more than half of the activity was lost, and no activity remained at 60°C.

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                                  ▼   ▼
  1 MFKFKKNFLVGLTAALMSISLFSATASAASTDYWQNWTDGGGTVNAVNGS

 51 GGNYSVNWSNTGNFVVGKGWTKGSPSRTINYNAGVWAPNGNGYLALYGWT
        *
101 RSPLIEYYVVDSWGTYRPTGTYKGTVYSDGGTYDIYTTKRYNAPSIEGQH
                                                      *
151 STFTQYWSVRRSKRPTGNNAKITFSNHVKAWKSHGMNLGSIWSYQVLATE

201 GYQSSGSSNVTVW
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Fig. 1 Amino acid sequence of MS514-Xyn11 enzyme. Underlined region denotes conserved GH11 xylanase domain. Starred amino acids are predicted essential catalytic residues. Arrowheads indicate putative cleavage sites of signal peptide

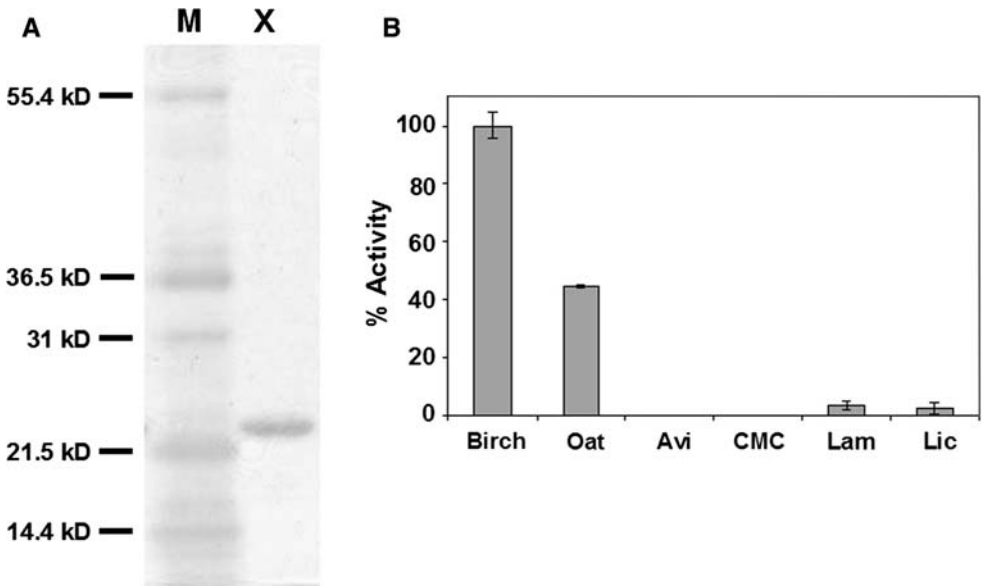

Fig. 2 (**a**) Purified MS514-Xyn11 enzyme. M, molecular weight markers; X, purified enzyme. (**b**) Relative enzymatic activity against various carbohydrate substrates. Birch, birchwood xylan; Oat, oat spelt xylan; Avi, Avicel; CMC, carboxylmethyl cellulose; Lam, laminarin; and Lic, lichenan

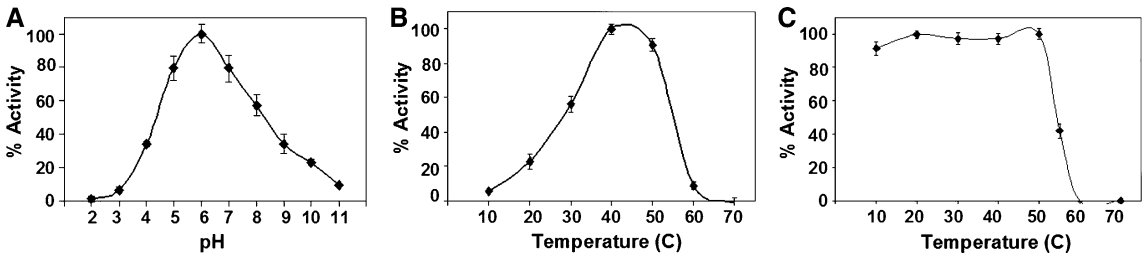

Fig. 3 pH and temperature activity profile. (**a**) Relative activity at 50°C and various pHs. (**b**) Relative activity at pH 6 and various temperatures. (**c**) Stability of enzyme after 30 min preincubation at various temperatures, followed by activity determination at 50°C and pH 6. All reactions used a 1% birchwood xylan substrate

The MS514-Xyn11 enzyme retained xylanase activity in the presence of a wide variety of cations (Table 1). Activity was stimulated by increasing the amounts of calcium, lithium, and magnesium. Cobalt, manganese, and zinc stimulated MS514-Xyn11 activity at lower concentrations but inhibited activity at the highest concentration tested (20 mM). Iron had little effect on the enzyme activity at lower concentrations but did inhibit at the highest concentration tested. Copper at 20 mM caused the most dramatic inhibition by reducing the activity to 12% of the control.

Kinetic parameters were calculated for the enzyme against birchwood xylan. The apparent K_m and V_{max} were determined to be 6.7 ± 0.7 mg/mL and 379 ± 15 μmol/min/mg, respectively. The products of the enzymatic digest were analyzed by capillary electrophoresis and found to be primarily xylopentaose, xylotetraose, and xylotriose. This pattern is consistent with that expected from an endoxylanase enzyme.

In summary, we have cloned a gene from *B. licheniformis* that encodes a xylanase enzyme, MS514-Xyn11. The enzyme was biochemically characterized and found to have no detectable cellulase activity and tolerance to a wide variety of cations. Thus, the MS514-Xyn11 enzyme could be useful as an additive to mixtures designed to hydrolyze biomass as well as in applications for which it is desirable to maintain cellulose structure.

Table 1 Effect of various cations on xylanase activity

	% No additive activity		
	5 mM	10 mM	20 mM
$CaCl_2$	116	109	131
$CoSO_4$	116	126	116
$CuSO_4$	120	28	12
$FeSO_4$	105	107	73
LiCl	125	122	138
$MgCl_2$	114	127	157
$MnSO_4$	127	140	75
$ZnSO_4$	125	93	79

All reactions were conducted at 50°C and pH 6 with 1% birchwood xylan. A reaction with no additive was assigned 100% activity. All other reaction rates in the table are relative to this standard

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